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BIODEGRADATION OF ENDOSULFAN BY MIXED BACTERIA CULTURE STRAINS OF *PSEUDOMONAS AERUGINOSA* AND *STAPHYLOCOCCUS AUREUS*

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ABSTRACT
Endosulfan is a synthetic cyclodiene non-systemic insecticide and acaricide with both contact and stomach activity. Microorganisms play a key role in removal of xenobiotics like endosulfan from the contaminanted sites because of their dynamic, complex, and complicated enzymatic systems which degrade these chemical. In this study, endosulfan was degraded by indigenous bacteria strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The biodegradation process was carried out according to the method established by Buff *et al*., 1992. The results obtained showed that the bacterial strains efficiently degraded endosulfan by consuming more than 90 % of 40 mg/l of the minimal media within 7 days of incubation. Maximum biodegration by these two selected bacterial stains was observed at an initial pH of 8.0 at an incubation temperature of 30 °C. The findings from present study suggest that these bacterial strains could be potentially promising as a low cost effective technology in the biodegradation of endosulfan from the environment.

Keywords: Endosulfan, Microoganisms, Biodegradation, Contamination and Soil.

INTRODUCTION
Intensification of agriculture and manufacturing industries has resulted in increase in release of a wide range of xenobiotics compounds to the environment. This extensive utilization of synthetic pesticides and / or agrochemicals results in the accumulation of the residues of these toxic chemicals in the soil (Kumar *et al*., 2007; Kumar *et al*., 2008; Kumar *et al*., 2013). These accumulated residues deteriorate the overall quality of the soil and that of the surrounding environment as well. Aside from this, pesticide-formulating industries are also contaminating the environment through various activities (Qureshi *et al.,* 2009). Pesticide exposure has also been shown to inflict chronic and acute threats to human health, and long-term low-dose exposure to pesticides causes immune suppression, hormonal disruption, diminished intelligence, reproductive abnormalities, and carcinoma (Arnold *et al*., 1996; Awasthi *et al*., 2003; Gupta, 2004).
Soil biodegradation is an option that offers the possibility to degrade or render various contaminants harmless using natural biological activity (Awasthi *et al*., 2000; Muhammad *et al*., 2016). Two important approaches of bioremediation are bioaugmentation and biostimulation. Biostimulation involves modification within the environment to stimulate existing bacteria capable of bioremediation (Ying-Hua *et al*., 2016). This can be done by addition of various forms of rate limiting nutrients and electron-acceptors, such as phosphorus, nitrogen, oxygen, or carbon (e.g. in the form of molasses). Additives are usually added to the subsurface through injection wells, although injection well technology for biostimulation purposes is still emerging. Removal of the contaminated material is also an option, albeit an expensive one. Biostimulation can also be enhanced by bioaugmentation (Martens, 1976). The primary advantage of biostimulation is that bioremediation will be undertaken by already present native microorganisms that are well-suited to the subsurface environment, and are well distributed spatially within the subsurface (Wang, 2012). On the other hand, Bioaugmentation involves the addition of highly concentrated and specialized populations of specific microbes indigenous or exogenous to a contaminated site to enhance the rate of contaminant biodegradation in the affected soil or water. Bioremediation through bioaugmentation is a promising, innovative, and low cost-effective technology for use in the cleanup of hazardous wastes. In this process, microorganisms transform environmental contaminants into harmless end products (Harishankar *et al*., 2013).
Although extensive research on the use of bioremediation as a means of detoxifying the environment has been carried out, there remains the need to investigate the effect of mixed bacteria culture strains on the biodegradation of pesticides. This study employs the use of *Pseudomonas aeruginosa (P.aeruginosa)* and *Staphylococcus aureus (S. aureus)* for the degradation of pesticide Endosulfan. Endosulfan which is a poor biological energy source, as it contains only six potential reducing electrons and previous attempts to enrich for endosulfan-degrading microorganisms using the insecticide as a carbon source have been unsuccessful. However, endosulfan has a relatively reactive cyclic sulfite diester group (Goebel *et al*., 1982). In this study, microorganisms were selected for their ability to release the sulfite group from endosulfan and to use this as a source of sulfur for growth. This selection procedure enriches for a culture capable of either the direct hydrolysis of endosulfan or the oxidation of the insecticide (Guerin and Kennedy, 1992; Guerin, 1999). However, the accumulation of endosulfate (the end product of endosulfan oxidation) and the inability of the bacterial to grow when endosulfate is provided as the sole sulfur source, will indicate from our analysis whether we have selected for the direct hydrolysis of endosulfan or not.

MATERIALS AND METHOD

Materials

The reagents used were purchased from Sigma Aldrich (Germany); they include NaOH, K_2HPO_4, KH_2PO_4, $MgCl_2$, $CaCO_3$, NH_4Cl, NaCl and Endosulfan salts.

Apparatus

A Jenway 6405 Spectrophotometer,a HACH DR 1890 Colorimeter, a Gallenkamp Autoclave, a Fisher Scientific 842 VES Centrifuge and a Jenway 3505 pH meter were used for the analysis carried out in this work.

Methods

Collection of Bacteria Strains

Bacteria strains used for this study were obtained from The Department of Microbiology (Ahmadu Bello University, Zaria).

Preparation of 1250 media Stock

Distilled water was used in the preparation of all aqueous solutions. Solutions prepared were stored in amber coloured bottles. Exactly 1250 cm^3 stocks of minimal media (non- sulfur medium) was prepared in 2000 cm^3 volumetric flask consisting of 1.25 g of K_2HPO_4, 1.25g of KH_2PO_4, 1.25 g of $MgCl_2.6H_2O$, 1.25 g of $CaCO_3$, 6.25 g of NH_4Cl, 1.25 g of NaCl, and 6.25 g of Yeast extract. The weighed salts were transferred into a 2000 cm^3 volumetric flask with continuous swirling for proper mixing and then made up to the mark with distilled water.

Bacterial Innovation Assay

Bacteria Innovation assay was carried out between the bacterial strains and Endosulfan. Exactly 250 cm^3 of the minimal media and 10 mg/l of Endosulfan were transferred into a 250 cm^3 conical flask. The conical flask and contents were sterilized for about 15 minutes in an autoclave (Gallenkamp, Japan) after which, it was allowed to cool to room temperature. The flask was then inoculated with mixed bacterial strains of *S.aureus* and *P.aeruginosa* ; plugged with sterile cotton wool and incubated at 30 ^oC in an incubator. A control experiment consisting of the minimal media and endosulfan only (without inoculate) was also set up. Every 24 hours, both the sample and blank were centrifuged and the supernatant transferred into a 1.0 cm glass cuvette and its absorbance determined at a wavelength of 400nm using ultraviolet/visible spectrophotometer (Jenway 6405, UK) to determine the level of biodegradation.

Biodegradation of Endosulfan at Variable Endosulfan Concentration

This was carried out according to the method of Buff *et al*., 1992. In this method biodegradation of endosulfan at varying endosulfan concentrations was studied using a 250 cm^3 Erlenmeyer flask. For this purpose, 250 cm^3 of already prepared minimal media was transferred into six different 250 cm^3 flasks. The flasks were enriched with different endosulfan concentrations of 20, 30, 40, 50, 60 and 70 mg/l. The pH of the media was adjusted to 8.0.; the flasks were plugged with sterile cotton wool and then covered with aluminum foil. The flasks and its content were sterilized in an autoclave (Gallenkamp, Japan), for about 15 minutes; after which, the conical flasks were removed from the autoclave and allowed to cool to room temperature. The media in the flasks were inoculated with aid of flamed sterilized wire loop with mixed bacterial strain of *P.aeruginosa* and *S.aureus* and then incubated at 30 ^oC in an incubator. Uninoculated flasks were also prepared to serve as control.

The bacteria growth in the media was evident by the degree of turbidity of the solution. At 24 hours interval, both the samples (inoculated flasks and uninoculated flasks) were centrifuged. The supernatant was transferred into a 1.0 cm glass cuvette and its absorbance was taken at a wavelength of 400 nm using spectrophotometer (Jenway 6405, UK) to determine the level of biodegradation.

The Effect of pH on Biodegradation of Endosulfan using *Pseudomonasaeruginosa* and *Staphylococcusaureus*

Exactly 150cm^3 of the media (enriched with endosulfan) was transferred into seven different conical flasks and the pH adjusted with NaOH to the pH value of 4, 5, 6, 7, 8, 9, and 10 using a pH meter (Jenway, UK). The flasks and its contents were autoclaved at 121^oC for about 15 minutes and then inoculated with the aid of flamed sterilized wire loop with mixed bacterial strain of *P.aeruginosa* and *S.aureus*; plugged with sterile cotton wool and incubated at a temperature of 30 ^oC. Also, uninoculated flasks were prepared to serve as blanks. The bacteria growth in the media was measured by taking the optical densities of the supernatant at a 24 hour interval for seven days.

Effect of Temperature on Biodegradation of Endosulfan

Exactly 250 cm^3 of the already prepared minimal medium was adjusted to a pH value of 8.0 and the endosulfan concentration of 40 mg/l were distributed into seven different flasks; the flasks were sterilized and then inoculated with the aid of flamed sterilized wire loop with mixed bacterial strain of *P.aeruginosa* and *S.aureus*. The flasks were plugged with sterile cotton wool and were incubated at various incubator temperatures of 20, 25, 30, 35, 37, 40, 45 ^oC for seven days. Uninoculated flasks were equally prepared to check abiotic degradation.

The bacterial growth in the media was monitored by the degree of turbidity of the solution at a 24 hour intervals with absorbance taken for the supernatant of both inoculated and uninoculated flasks at a wavelength of 400 nm using a spectrophotometer (Jenway6405, UK) to determine level of biodegradation.

The amount of Sulfate released during the process of Endosulfan degradation was equally determined using a HACH Colorimeter.

RESULTS AND DISCUSSION

The degradation of Endosulfan by *P. aeruginosa* and *S. aureus* was determined at different concentrations by measuring its disappearance over an incubation period of 7 days. Figure 1 revealed the degradation of Endosulfan under inoculated conditions.

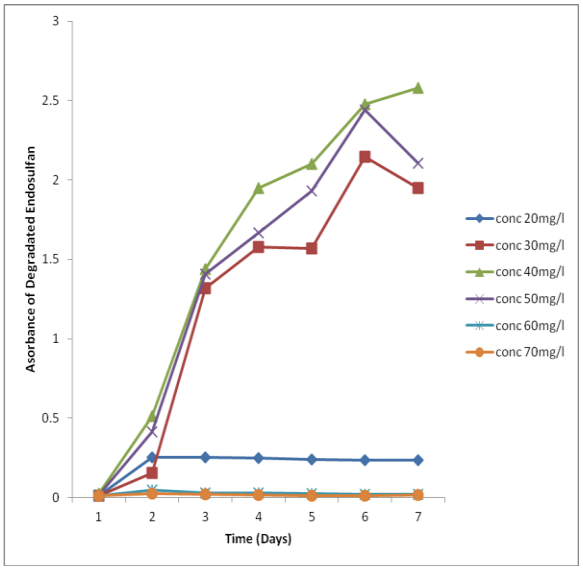

Figure 1: Growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus* with time at varying concentration of Endosulfan**.**

The growth of *P. aeruginosa* and *S. aureus* was monitored by measuring the absorbance of the culture at a wavelength of 400 nm at different time intervals. In the case of concentration of 20 mg/l, the curve was almost linear, indicating no significant increase in optical density with time; whereas substantial increase in growth of *P.aeruginosa* and *S.aureus* was observed at concentration of 30 mg/l, 40 mg/l and 50 mg/l. Maximum increase in bacterial growth was recorded at concentration of 40 mg/l, implying maximum biodegradation of endosulfan. Further increase in endosulfan concentration resulted in little or no reasonable growth of the bacteria (linear curve). This is because as the concentration of endosulfan increase above 60 mg/l, it becomes toxic to the bacteria thereby inhibiting its ability to degrade (Ying-Hua *et al*., 2016). Degradation was achieved and maintained by providing endosulfan as the only sulfur source (Buff *et al*., 1992). Endosulfan is degraded by an attack at the sulfide group via both oxidation and hydrolysis to form the toxic endosulfan sulfate and the non-toxic endosulfandiol respectively (Singh *et al*., 2004). The formation of endosulfate is thought to occur only through biological transformation, whereas hydrolysis to the diol occurs readily at alkaline pH (Bhalerao and Puranik, 2007). But the formation of intermediate compounds (metabolites) is mainly based on the metabolic activity of the specific culture and the environmental conditions (Zhang *et al*., 2014).
Biodegradation of endosulfan by efficient bacterial strains *P.aeruginosa* and *S.aureus* was further investigated at different initial pH of culture media; in an attempt to examine the role of pH in the biodegradation endosulfan. Experiments were designed using sterile minimal media at different pH values and results are presented in Figure 2.

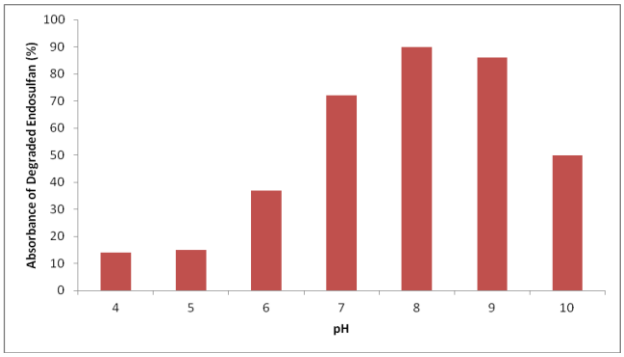

Figure 2: Growth of Bacteria at Different incubation pH

Biodegradation of endosulfan by these bacterial cultures was more pronounced at alkaline pH values of the culture media and was strongly inhibited at acidic pH values. Biodegradation of endosulfan by *P.aeruginosa* and *S.aureus* in minimal media at initial pH 4 to 10 ranged from 14 to 90%. The results further revealed that biodegradation significantly increased with increase in pH up to 8. At pH 8 there was a 90% biodegradation of endosulfan. Minimum biodegradation was recorded at pH 4 and pH 5. Thus, we can conclude that at pH of 8 we expect 90% biodegradation of endosulfan.

Biodegradation of endosulfan by *P.aeruginosa* and *S.aureus* was also investigated at various incubation temperatures and the result presented in Figure 3.

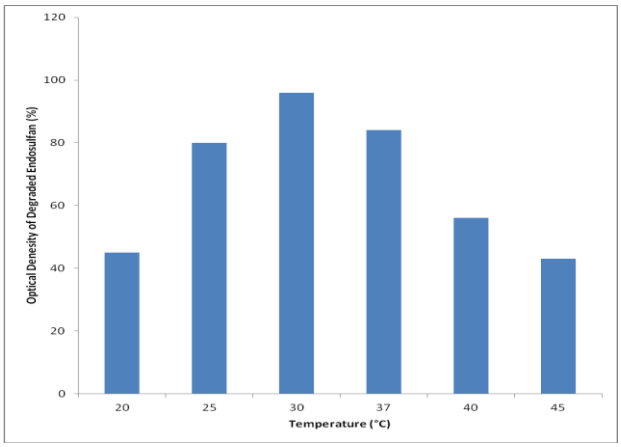

Figure 3: Bacterial Growth with Different Incubation Temperature

Biodegradation of Endosulfan was relatively greater as incubation temperature ranges from 25 °C to 37 °C with a maximum at 30 °C and a minimum temperature of 45 °C.

Endosulfan has a relatively reactive cyclic sulfite diester group (Awasthi *et al*., 2003). In this study, microorganisms were selected for their ability to release the sulfite group from endosulfan. This released sulfite is oxidized to sulfate; the amount of sulfate released was high and determined to be 80mg/l which indicated that endosulfan in this study was degraded to a non-toxic substance endosulfandiol. The reaction pathway and the intermediates likely to be form in the process of endosulfan degradation are shown in Figure 4 below

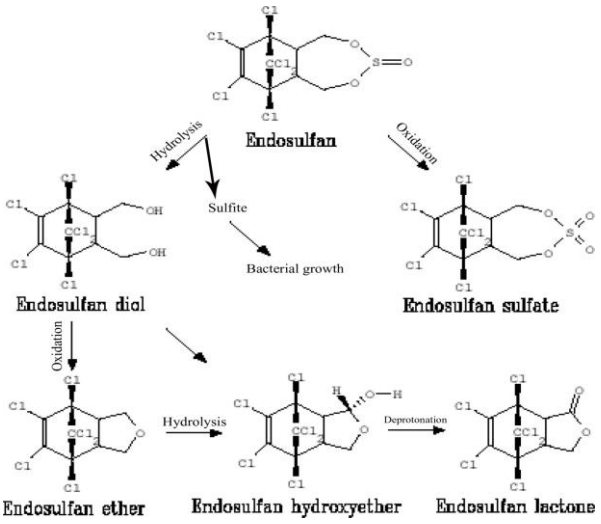

Figure 4: Reaction Pathway and the Intermediates Formed (Arnold *et al*., 1996)

Conclusion

The goal of any bioremediation study is to ensure that harmful substances are broken down into more environmentally friendly substances. This no doubt will ensure that our environment is in a healthy state and its products safe for human consumption. In this study, we have found that the bacterial strains *P.aeruginosa* and *S.aureus* showed a high efficiency of endosulfan degradation with initial endosulfan concentration of 40 mg/l, at an optimal pH of 8.0 and incubation temperature of 30 °C. This indicates that the bacteria strains of *P. aeruginosa* and *S. aureus* may be used as a potential source of hydrolyzing enzymes in bioremediation of damaged habitats. Thus, apart from its use in bioremediation processes, bacteria strains could be potentially promising as low cost effective technology in the biodegration of endosulfan from the environment.

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